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The gene of the nuclear receptor coactivator AIB1 is amplified in breast cancer cell lines as well as in breast tumor tissue. AIB1 mRNA is often highly expressed in primary breast tumors and is has been shown that AIB1 enhances estrogen and progesterone dependent transcription *in vitro*. Therefore it has been postulated that AIB1 contributes to the development of breast cancer. However, it is currently not known what the precise role of AIB1 is in the development of breast cancer. To address this question we established MCF-7 breast cancer cell lines in which we can regulate AIB1 levels with ribozymes in order to determine the impact of reduced AIB1 gene expression on the phenotype and angiogenic or invasive properties of breast cancer cells. Here we report that depletion of endogenous AIB1 levels reduced steroid hormone signaling via the estrogen receptor-alpha or progesterone receptor-beta as well as estrogen-mediated inhibition of apoptosis and cell growth. Furthermore, we demonstrate that upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MCF-7 cells in nude mice was decreased. We conclude that AIB1 exerts a rate-limiting role for hormone dependent human breast tumor growth.

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Introduction

Small fat soluble hormones such as steroids, retinoids and vitamin D_3 play pivotal roles in the control of breast cancer proliferation and differentiation. The biological effects of these molecules are mediated through intracellular receptor proteins. Estrogens stimulate proliferation of estrogen receptor (ER) positive breast cancer cells and the ER status of a breast tumor is predictive of the outcome of the disease. Therapy (such as tamoxifen) targeted at reducing the estrogenic stimulus to the breast has been shown to be effective. Conversely retinoids and vitamin D_3 are strongly growth inhibitory in breast tumor cells and analogues of these compounds are currently being tested for efficacy in breast cancer. Recently the discovery of proteins known as steroid receptor co-activators has led to another level of complexity to our understanding of how hormones exert their effects [1,2]. Of particular interest is the co-activator **AIB1 (amplified in breast cancer 1)**, which interacts with the estrogen receptor (ER) [3]. Interestingly, the AIB1 gene was found to be amplified and AIB1 expression is increased in breast tumors and breast cancer cell lines [3,4,5]. **However, it is currently not known what the role of AIB1 amplification is in the development of breast cancer nor is it known whether detection of AIB1 overexpression will be valuable for diagnosis of breast cancer or for prognosis of disease outcome.** The possible role of AIB1 in breast cancer is further complicated by studies which demonstrate that AIB1 can also interact with retinoid, thyroid, Vit D_3, PPAR and androgen receptors [6-8]. **This broad ability to potentiate the effects of a number of hormone receptors leads to the question of what is the precise role of the AIB1 amplification in breast cancer?**

In this proposal we will investigate the hypothesis that overexpression of AIB1 in breast cancer is important for breast tumor development by impacting upon nuclear hormone receptor function. In particular we wish to investigate if AIB1 is required for expression of a gene(s) critical for breast cancer development. The novel approach we will take to study this question will be to develop hammerhead ribozymes to target and cleave AIB1 thus producing a selective reduction of this coactivator in breast cancer cells. Ribozymes are molecules of RNA which can cleave a specific target RNA (in this case AIB1) and thus selectively reduce expression of the AIB1 protein in the cell. In addition, the potential development of ribozymes as potent therapeutic agents [9] makes the translation of these results into possible therapies realistic. **In this study we will examine the idea that the nuclear receptor coactivator AIB1 is rate-limiting for breast cancer development and that selective targeting of this coactivator will be useful for future development of novel therapies leading to reduced proliferation or metastatic potential of these cells.** Initially our experiments have been focued on designing ribozymes which selectively decrease AIB1 *in vivo* (see first Annual Report 2000). Using these reagents as tools, we will now determine the impact of reduced AIB1 gene expression on estrogen, progesterone, retinoid, Vit D_3, PPAR and AR receptor function in breast cancer cell lines. Finally we will determine if reduction of AIB1 mRNA can influence the progression of breast cancer *in vivo*. **We anticipate that these experiments will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.**

Proposal Body

In the approved Statement of Work three Tasks were outlined.

Task 1: Development of AIB1 selective targeted ribozymes (month 1-12)

Task 2: Determining the impact of reduction in AIB1 mRNA on the phenotype of breast cancer cells (6-30 month)

Task 3: To determine if the reduction in AIB1 mRNA alter the angiogenic or invasive properties of breast cancer cell lines (12-36 month)

The following report about our progress includes data showing progress mainly concerning Task 2. Specifically:

Task 1: i) The first goal of our studies was the development of a series of plasmids that will specifically target AIB1 mRNA in vectors with the CMV promoter and also in vectors with the regulatable tetracycline promoter.

We completed Task 1 during the first year of the funding period. In summary, we were able to i) design, construct and transfect several plasmids containing regulatable AIB1 ribozymes and ii) to obtain stably transfected MCF-7 cell lines in which AIB1 protein levels were reduced by up to 90% compared to wild type cells (see first Annual Report from 2000).

Task 2: The goals of our studies regarding Task 2 were i) to determine the impact of reduction of AIB1 mRNA on gross phenotype changes in cells including proliferation, differentiation and apoptosis and ii) to determine the effect of AIB1 reductions on gene expession and promoter activity of individual hormones.

Rate-limiting role of AIB1 for MCF-7 breast cancer cell growth

In order to analyze the influence of AIB1 on the phenotype of human breast cancer cells we performed growth assays and soft agar colony formation assays with MCF-7 cells expressing regulatable AIB1 ribozymes (development of these cell lines was described in the first Annual Report; see also Task 1). We found in cell proliferation assays that reduction of endogenous levels of AIB1 in MCF-7 breast cancer cells reduced estrogen-dependent growth of these cells (Appendix: Manuscript Fig. 4). In addition, estrogen-dependent colony formation of MCF-7 cells was strongly reduced after downregulation of AIB1 (Appendix: Manuscript Fig. 7). Furthermore, when we tested tumor growth of MCF-7 cells in nude mice we could demonstrate that reduction of

AIB1 levels significantly decreased tumor growth of these cells in nude mice (Appendix: Manuscript Fig. 8). In summary, we were able to demonstrate that the nuclear coactivator AIB1 exerts a rate-limiting role for hormone-dependent human breast cancer cell growth.

Role of AIB1 for cell cycle progression and apoptosis in MCF-7 cells

Since estrogens contribute to cell cycle progression and inhibition of apoptosis in MCF-7 cells, we analyzed whether slower estrogen-mediated growth after AIB1 downregulation might have resulted from a reduced ability of these cells to progress through the cell cycle or whether this effect might have been based on their altered susceptibility towards apoptosis. While we found no changes in cell cycle progression of MCF-7 cells after downregulation of AIB1 (Appendix: Manuscript Fig. 5), we could demonstrate that the ability of these cells to inhibit apoptosis after estrogen stimulation was strongly reduced in cells with lower AIB1 levels, indicating that the reduction of estrogen-mediated cell growth of MCF-7 cells expressing the AIB1 ribozyme is at least partially due to the reduced ability of estrogen to inhibit apoptosis.

Effect of AIB1 reduction on estrogen and progesterone responsive promoters

Here we tested whether AIB1 levels influenced the inducibility of estrogen and progesterone responsive promoters in MCF-7 cells. Our data showed that reduction of endogenous AIB1 levels in MCF-7 cells lead to a reduction of estrogen and progesterone responsiveness of a estrogen/progesterone-responsive test promoter (Appendix: Manuscript Figs. 1 & 3). We could therefore demonstrate that AIB1 is necessary for full transcriptional activation of hormone-responsive promoters *in vivo*.

Task 3: The goals of our studies regarding Task 3 were to determine if the reduction in AIB1 mRNA alter the angiogenic or invasive properties of breast cancer cell lines.

These goals will be mainly performed in year three of the funding period. So far, during this funding period, we were able to set up a modified Boyden Chamber assay for MCF-7 cells which will allow us to investigate the influence of AIB1 on cell motility and invasive behavior.

A detailed description of our findings is found in the manuscript 'Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MFCF-7 breast cancer cells', supplied in the appendix of this report.

Key Research Accomplishments

- We demonstrated that AIB1 is necessary for full transcriptional activation of the estrogen receptor alpha and the progesterone receptor beta *in vivo*.

- We showed that AIB1 is rate-limiting for estrogen-dependent cell growth in human MCF-7 breast cancer cells.

- We showed that downregulation of endogenous AIB1 levels in MCF-7 cells did not affect estrogen-stimulated cell cycle progression but reduced estrogen-mediated inhibition of apoptosis.

- We demonstrated that upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MFCF-7 cells in nude mice was decreased.

Reportable Outcomes

- List, H.-J., Lauritsen, K.J., Reiter, R., Powers, C., Wellstein, A. and Riegel, A.T. (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MFCF-7 breast cancer cells. J. Biol. Chem. 276, 23763-23768.

Conclusions

In this study we want to examine whether the nuclear receptor coactivator AIB1 is rate-limiting for breast cancer development and whether selective targeting of this coactivator will be useful for the future development of novel therapies leading to reduced proliferation or metastatic potential of these cells. Specifically, we want to answer the question of "what is the precise role of AIB1 for breast cancer development *in vivo* considering its broad ability to potentiate the effects of a number of hormone receptors *in vitro*"?
During the first year of this project we have developed MCF-7 breast cancer cell lines in which we were able to regulate AIB1 protein levels by targeting AIB1 with regulatable ribozymes specifically directed against AIB1 mRNA. During this funding period, utilizing these cell lines, we could demonstrate that the nuclear receptor coactivator AIB1 is rate-limiting for estrogen-dependent growth, colony formation in soft agar and tumor growth of MCF-7 cells in nude mice. From our findings, we conclude that despite the presence of different estrogen receptor coactivators in breast cancer cells, AIB1 exerts a rate-limiting role for hormone-dependent human breast tumor growth. We therefore anticipate that the proposed experiments, outlined in the approved Statement of Work, will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.

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Ribozyme Targeting Demonstrates That the Nuclear Receptor Coactivator AIB1 Is a Rate-limiting Factor for Estrogen-dependent Growth of Human MCF-7 Breast Cancer Cells*

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Human breast tumorigenesis is promoted by the estrogen receptor pathway, and nuclear receptor coactivators are thought to participate in this process. Here we studied whether one of these coactivators, AIB1 (amplified in breast cancer 1), was rate-limiting for hormone-dependent growth of human MCF-7 breast cancer cells. We developed MCF-7 breast cancer cell lines in which the expression of AIB1 can be modulated by regulatable ribozymes directed against AIB1 mRNA. We found that depletion of endogenous AIB1 levels reduced steroid hormone signaling via the estrogen receptor α or progesterone receptor β on transiently transfected reporter templates. Down-regulation of AIB1 levels in MCF-7 cells did not affect estrogen-stimulated cell cycle progression but reduced estrogen-mediated inhibition of apoptosis and cell growth. Finally, upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MCF-7 cells in nude mice was decreased. From these findings we conclude that, despite the presence of different estrogen receptor coactivators in breast cancer cells, AIB1 exerts a rate-limiting role for hormone-dependent human breast tumor growth.

Human breast tumorigenesis is promoted by enhanced activity of the estrogen receptor (ER)[1] pathway. It has been shown that estrogens can directly cause proliferation of breast cancer cells (1) and that more than 70% of primary human breast cancers are ER-positive. The activity of the ER is modulated by a recently discovered class of specific corepressors and coactivators that inhibit or enhance the transcriptional activity of the ER as well as related nuclear hormone receptors (2–4). In the absence of ligand, some of the nuclear receptors are bound to corepressors such as SMRT and NCoR (5, 6). After ligand binding, the corepressors are released, and nuclear receptor coactivators are recruited. This leads to the enhancement of transcriptional activity of the nuclear receptor via interaction with chromatin remodeling complexes and members of the basal transcription machinery (2, 3).

Some of the best characterized nuclear receptor coactivators belong to the p160/steroid receptor coactivator (SRC) family. In humans, this family consists of SRC-1 (7), TIF-2 (8), and AIB1 (9) (ACTR/RAC3/TRAM-1/SRC-3) (10–13). Special attention has been focused on the gene AIB1 (amplified in breast cancer 1), which is amplified in breast, ovarian, pancreatic, and gastric cancer (9, 14, 15). Amplification of the AIB1 gene was detected in 5–10% of primary breast tumors, and AIB1 mRNA was found to be highly expressed in many breast tumor specimens (9, 16–18). Furthermore, AIB1 amplification correlates with estrogen and progesterone receptor positivity of primary breast tumors as well as with tumor size (16, 19). AIB1 binds directly to ER *in vivo* (20) and enhances *in vitro* the transcriptional activity of the estrogen receptor (9, 10, 13) as well as a number of other nuclear receptors, including the progesterone, thyroid hormone, and retinoid acid receptor (10–12). In addition, it has been shown that AIB1 interacts with other transcription factors such as TEF (21) and NF-κB (22) and that AIB1 inhibits p53-dependent transactivation (23). Interestingly, a recent study demonstrated that AIB1 overexpression is correlated with the absence of ER and PR but is positively correlated with the expression of p53 and HER2/neu, indicating that in a subset of breast tumors AIB1 might also be involved in signaling pathways other than for steroid hormones (24). p/CIP, the mouse homolog of AIB1, is required for CBP-dependent transcriptional activation induced by interferon-γ and 12-*O*-tetradecanoylphorbol-13-acetate (25). Disruption of p/CIP results in a pleiotrophic phenotype including reduced female reproductive function and blunted mammary gland development in mice as well as in the production of endogenous estrogen (26). In addition, p/CIP also seems to play a role for the expression of genes critical for somatic growth and in several growth hormone regulatory pathways (27). Taken together, these findings led to the hypothesis that human AIB1 contributes to the development of breast cancer, but evidence that AIB1 directly affects breast cancer growth and development is still lacking.

In this study, we investigated the function of AIB1 for breast cancer cell proliferation, apoptosis, and tumor growth in mice. We selected the well characterized human breast cancer cell line MCF-7 for our studies, since it was shown earlier that these cells express high levels of AIB1 protein (13, 18). In addition, AIB1 interacts with the endogenous estrogen receptor in these cells (20), enabling us to investigate the role of AIB1 for estrogen-dependent growth. We now report that reduction of endogenous AIB1 levels in MCF-7 cells by ribozyme-targeting reveals a significant role of this coactiva-

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[1] The abbreviations used are: ER, estrogen receptor; SRC, steroid receptor coactivator; PR, progesterone receptor; IMEM, Iscove's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline.

tor for estrogen-dependent growth and apoptosis as well as for tumor growth in mice.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 cells were cultivated in IMEM (Life Technologies) supplemented with 10% FCS (Life Technologies, Inc.). MCF-7 cells that were stably transfected with ribozyme expression vectors were cultivated in IMEM supplemented with 10% FCS, 400 μg/ml G418 (Invitrogen), and 400 μg/ml zeocin for MCF-7/Rz12 (Invitrogen) or 1 μg/ml puromycin for MCF-7/Rz23 (Sigma) in the presence or absence of 1 μg/ml doxycycline (Sigma).

Ribozyme Expression Vectors and Generation of Stable Cell Lines—To generate the ribozyme constructs for transient transfections, synthetic sense and antisense oligonucleotides containing the catalytic center and flanking regions of the ribozymes as well as AIB1 homologous regions were annealed and ligated into the *Hin*dIII/*Not*I sites of pRc/CMV (Invitrogen). The sequences for the upper strand oligonucleotide were 5′-AGCTTGAATCGATACTGATGAGTCCGTTAGGACGAAACTGGGGTTGC-3′ for ribozyme 12 and 5′-AGCTTAGAACTACCTGATGAGTCCGTTAGGACGAAACACCTGAAGC-3′ for ribozyme 23. Ribozyme-expressing cell lines were obtained by co-transfection of MCF-7 cells, which stably express the tetR-VP16 transactivator protein (28), with ribozyme expression vectors that had been constructed by insertion of the synthetic ribozymes (see above) into the tetracycline-regulatable vector pTET (29) and pSV2 NEO (CLONTECH) (MCF-7/Rz12) or pBabePuro (30) (MCF-7/Rz23). The cells were selected in IMEM plus 10% FCS supplemented with G418 (400 μg/ml), zeocin (400 μg/ml), or puromycin (1 μg/ml) in the presence of doxycycline (1 μg/ml) for 4–6 weeks. Individual clonal cell lines were obtained by selection following transfection of the cells.

RNA Preparation and Northern Blot Analysis—For the preparation of cytoplasmic RNA, 70–80% confluent cells were lysed in ice-cold lysis buffer (0.2 M Tris-HCl, pH 8.0, 140 mM NaCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40). After incubation for 4 min on ice, the mix was centrifuged at 12,000 rpm at 4 °C. Cytoplasmic RNA was obtained by extraction with STE buffer (5 mM Tris-HCl, pH 8.5, 2 mM EDTA, 0.2% SDS) and phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). Northern blot analysis was performed with 15 μg of cytoplasmic RNA using a radiolabeled 0.75-kilobase pair *Eco*RI fragment from AIB1/ACTR for hybridization (10). AIB1 transcript levels were quantified by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). All AIB1 levels were corrected with glyceraldehyde-3-phosphate dehydrogenase for loading differences.

Western Blot Analysis—For Western blot analysis, 70–80% confluent cells were washed with PBS, harvested with a cell scraper, and washed once with PBS and twice with wash buffer (10 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, protease inhibitor mixture (Complete™; Roche Molecular Biochemicals)). The cell pellet was resuspended in lysis buffer (20 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, protease inhibitor mixture, 0.1% Nonidet P-40) and incubated for 10 min on ice. The suspension was centrifuged at 10,000 × *g* at 4 °C for 5 min. 40 μg of supernatant protein was electrophoresed on a 4–20% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the membrane was incubated for 1 h at room temperature with 5% nonfat milk in PBST (PBS, 0.05% Tween 20) followed by washing three times for 15 min each with PBST. The membrane was incubated for 1 h at room temperature with primary anti-AIB1 antibody (Transduction Laboratories), washed as described above, and incubated for 1 h with a secondary antibody-peroxidase conjugate (10,000-fold dilution in PBST). After washing, the membranes were incubated for 1 min with ECL detection solution (Amersham Pharmacia Biotech) and then exposed to film. Bands were quantitated using densitometry.

Transient Transfections—In order to measure transcriptional activation from an estrogen-responsive reporter (pERE-Luc), MCF-7 cells were plated at 60–70% confluence in IMEM plus 10% FCS in six-well plates (5 × 10^5 cells/well) 24 h prior to transfection. The cells were transfected with 1 μg of pERE-Luc harboring three copies of the *Xenopus* vitellogenin A_2 estrogen response element driving a luciferase reporter (pERE-Luc) (31), 0.1 ng of pRL-CMV *Renilla* luciferase reporter, and 3 μg of the AIB1 ribozyme expression vectors or control vector in 8 μl of LipofectAMINE™ (Life Technologies). After 5 h, the transfection mix was removed, and the cells were cultivated for 72 h in IMEM plus 10% FCS in the presence of 10 nM ICI 182,780 with or without 100 nM 17β-estradiol. In order to test progesterone activity, MCF-7 cells were plated in IMEM plus 1% charcoal-stripped fetal calf serum, transfected with 1 μg of a plasmid harboring the murine mammary tumor virus promoter (pMMTVLuc), 20 ng of pPRβ, 0.1 ng of pRL-CMV *Renilla* luciferase reporter, and 3 μg of the AIB1 ribozyme expression vectors or control vector, and cells were treated for 72 h with 1 nM R5020 or vehicle. Transfections with MCF-7 cell lines that stably expressed the AIB1 ribozymes were performed in the presence or absence of 1 μg/ml doxycycline and carried out for 24 h instead of 72 h. Cells were washed twice with PBS and resuspended in lysis buffer (0.1 M potassium phosphate buffer, pH 7.8, 0.1% Triton X-100, 100 mM dithiothreitol). After centrifugation, the luciferase assay and the correction for transfection efficiency were performed with 10 μl of supernatant as described previously (32).

Proliferation Assays—24 h before treatment, cells were plated in IMEM plus 10% FCS in 96-well plates (1,500–3,000 cells/well). The cells were then treated with IMEM plus 10% FCS containing 10 nM ICI 182,780 with or without 100 nM 17β-estradiol and cultivated with or without 1 μg/ml doxycycline for up to 6 days. Cell numbers were determined by a colorimetric assay, based on the cleavage of the tetrazolium salt Wst-1 in viable cells, according to the protocol of the manufacturer (Roche Molecular Biochemicals).

Cell Cycle Analysis—Cells were serum-starved in the presence of 10 nM ICI 182,780 for 48 h and then treated for 24 h with 10 nM ICI 182,780 in the absence or presence of 100 nM 17β-estradiol and harvested. Cell cycle analysis was performed by the Vindelov staining method as described (33). In short, 2 × 10^6 cells were resuspended in 100 μl of 40 mM citrate/Me_2SO buffer. After the addition of trypsin inhibitor and ribonuclease A for 10 min, the cells were stained with propidium iodide, and cell cycle analysis was performed by flow cytometry.

Apoptosis Assay—Cells (1 × 10^6) were serum-starved in the presence of 10 nM ICI 182,780 for 72 h and then treated for 48 h with 10 nM ICI 182,780 in the absence or presence of 100 nM 17β-estradiol and harvested. After washing, the cells were resuspended in 100 μl of propidium iodide-annexin V-fluorescein isothiocyanate dual staining solution according to the protocol of the manufacturer (Trevigen) and incubated in the dark for 15 min at room temperature. 400 μl of 1× binding buffer was added to the cell suspension, and cells were analyzed by flow cytometry within 1 h.

Soft Agar Colony Formation Assays—MCF-7/Rz23–9 cells suspended in 0.35% agar (20,000 cells/dish) were layered on top of 1 ml of solidified agar (0.6%) in a 35-mm dish in the presence or absence of 10 nM ICI 182,780 with or without 100 nM 17β-estradiol. IMEM growth medium with a final concentration of 10% fetal bovine serum was included in both layers with or without 1 μg/ml doxycycline. After 7–9 days of incubation, colonies with a diameter of ≥80 μm were counted with an image analyzer (Omnicon). Experiments were carried out in triplicate.

Tumor Growth in Nude Mice—Twenty million tumor cells (MCF7/Rz23–9 cells) suspended in 0.2 ml of PBS were injected subcutaneously into the flanks of athymic female nude mice. One day before injection, the mice received one estrogen pellet (0.72 mg/pellet 17β-estradiol; Innovative Research of America) and were fed with either a doxycycline containing diet (200 mg/kg doxycycline; Bioserv) or normal food throughout the study. Tumor growth was followed for 2 months by measuring the tumor area every 2–3 days.

RESULTS

Ribozyme Targeting of Endogenous AIB1 in MCF-7 Cells—For reduction of AIB1 levels in MCF-7 cells, we designed five different hammerhead ribozymes directed against different regions of AIB1 mRNA. Four ribozymes were directed against the translated region of the AIB1 mRNA and one against the 3′-untranslated region (Fig. 1*A*). We first screened for ribozyme activity by transiently transfecting MCF-7 cells with different ribozyme expression vectors. Based on previous observations that AIB1 increases nuclear receptor-mediated transcription in transient transfection assays (9–13), we predicted that down-regulation of AIB1 should decrease transcriptional activation by the ER, and we used this as a read-out for our initial assays. We transiently transfected the empty vector or each of the ribozyme expression vectors under the control of the cytomegalovirus promoter together with a luciferase reporter harboring an estrogen-responsive promoter (pERE-Luc). After 72 h of hormone induction, we found that two of our ribozyme expression vectors, pCMV-Rz12 and pCMV-Rz23, reduced estrogen-mediated activation of pERE-Luc by 20 and 25%, respectively

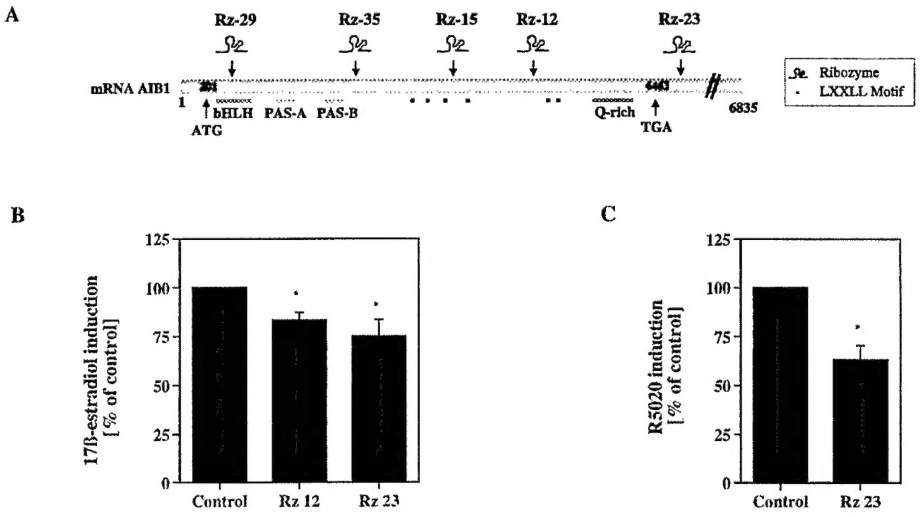

FIG. 1. **Transiently transfected AIB1 ribozymes reduce estrogen- and progesterone-mediated transcriptional activation.** *A*, ribozyme target sites in the mRNA of AIB1. The position of the helix-loop-helix domain (*bHLH*), the Per-Arnt-Sim domains A and B (*PAS-A* and *PAS-B*), a poly-Q rich region, various nuclear receptor-interacting domains (LXXLL motif), and the AIB1 translation start and stop sites are indicated. The target sites of the tested ribozymes are indicated. *B*, MCF-7 cells were transfected with an estrogen-responsive luciferase reporter (pERE-Luc), and empty vector or the pCMV-Rz12 or pCMV-Rz23 expression vector. The -fold induction by estradiol of control (empty vector)-transfected cells was set at 100%. *C*, MCF-7 cells were transfected with a progesterone-responsive reporter (pMMTVLuc) and pCMV-Rz23, and the effect of R5020 induction relative to that of cells transfected with an empty vector (control) is shown. The data are means ± S.E. from two (*B*) or three (*C*) independent experiments done in triplicate. *, $p < 0.05$ *versus* values from control transfected cells (Student's *t* test). For details, see "Experimental Procedures."

(Fig. 1*B*), indicating ribozyme activity. Other ribozyme expression vectors in the same vector backbone had no effect on the estrogen response (data not shown), indicating that the reduction of ER activity was due to the inserted ribozyme in the Rz12 and Rz23 constructs. To test whether the ribozyme effect was only on estrogen-mediated transcription or also affected transcriptional activation mediated by other hormones, we transiently transfected MCF-7 cells with a progesterone-responsive reporter (pMMTVLuc) together with the most effective ribozyme pCMV-Rz23. Consistent with the effect on ER signaling, we found a 35% reduction of the progesterone-mediated induction of transcription (Fig. 1*C*), indicating that endogenous AIB1 is involved in at least two hormone-mediated signaling pathways in MCF-7 cells and that these ribozymes could effectively regulate endogenous AIB1 levels in MCF-7 cells.

To study the influence of AIB1 on the proliferation of MCF-7 cells, we stably transfected MCF-7 cells with expression vectors for Rz12 and Rz23. For these experiments, we placed these ribozymes under the control of tetracycline-regulated expression vectors (pTETRz12 and pTETRz23, respectively; tet-off system). This system allowed us to specifically regulate AIB1-ribozyme expression (and hence endogenous AIB1 levels) by tetracycline or doxycycline withdrawal in isogenic cells and thus to avoid effects based on clonal selection. We transfected MCF-7 cells that stably expressed the tetR-VP16 transactivator with the ribozyme expression vectors and selected individual clones. To test the efficacy of the transfected ribozymes, we performed Northern and Western blot analysis of various clones that were obtained after 4–6 weeks of cultivation in selection media. We detected a 5–15% reduction of AIB1 mRNA and 25–40% of protein levels in clone MCF-7/Rz12–9 and a 30–50% reduction of mRNA and over 50% of protein for clone MCF-7/Rz23–9 (Fig. 2, *A* and *B*). These data demonstrate that we established two clonal MCF-7 cell lines containing regulatable ribozymes in which we can specifically down-regulate AIB1 levels.

Influence of Endogenous AIB1 on Steroid Hormone-dependent Transcriptional Activation—We tested whether down-regulation of endogenous AIB1 levels had any functional consequences for hormone signaling in these cells, as suggested by

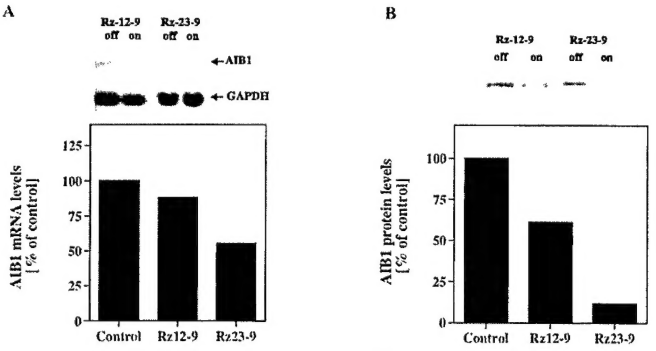

FIG. 2. **Reduction of endogenous AIB1 levels in MCF-7 cells.** *A*, Northern blot analysis of AIB1 from MCF-7 cells stably transfected with AIB1 ribozymes. The *top panel* shows a representative Northern blot from the clonal cell lines MCF-7/Rz12–9 and MCF-7/Rz23–9. Cells were cultivated in the presence (Rz off) or absence (Rz on) of doxycycline. RNA loading was corrected for by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels. Quantitation, shown in the *lower panel*, was done with a PhosphorImager. The AIB1 mRNA levels for doxycycline-treated cells were set arbitrarily as 100% for each cell line. *B*, Western blot analysis of AIB1 from cells, which were cultivated in the presence (Rz off) or absence (Rz on) of doxycycline. The *top panel* shows a representative Western blot. Quantitation, as shown in the *lower panel*, was done by densitometry, whereby the AIB1 levels for doxycycline-treated cells were arbitrarily set as 100%.

the transient transfection assays (Fig. 1). Indeed, cotransfection of the PR-sensitive pMMTVLuc and PR-β into the cell line MCF-7/Rz23–9 showed a strong reduction of PR-β activity induced with the synthetic progesterone R5020 in the cells which expressed the AIB1 ribozyme (Fig. 3*A*). As a negative control we used MCF-7 cells stably transfected with an empty vector (Fig. 3*B*). From this we concluded that in MCF-7 cells containing a tetracycline-regulated AIB1 ribozyme, reduction of AIB1 levels correlated with a reduction of progesterone-mediated transcriptional activation indicating a rate-limiting role for AIB1 in hormone signaling *in vivo*.

AIB1 Function in MCF-7 Breast Cancer Cell Growth—Based on the reduced ability of the ER and PR to activate the expression of a hormone-responsive reporter gene after down-regula-

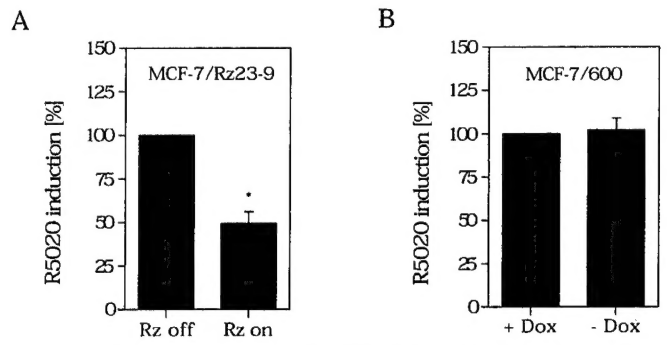

FIG. 3. **Stable AIB1-ribozyme expression reduces R5020-mediated transcriptional activation.** *A*, MCF-7/Rz23–9 cells were transfected with a progesterone-responsive luciferase reporter (pMMTVLuc) and a PR-β expression vector and treated with or without 1 nM R5020 for 24 h with or without doxycycline (*Dox*). The induction of luciferase activity by R5020 relative to control (+ *Dox* = Rz off) is shown. Mean ± S.E. from four independent experiments done in triplicate is shown. *, $p < 0.05$ *versus* values from doxycycline-treated cells (Student's *t* test). *B*, MCF-7/600 control cells, which contained no ribozyme expression vector, were transfected, treated with 1 nM R5020 in the presence or absence of doxycycline, and analyzed as described for *A*.

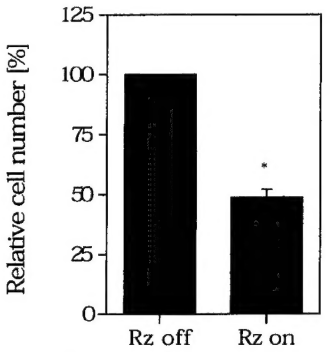

FIG. 4. **Estrogen-dependent growth of MCF-7 cells is reduced in AIB1 ribozyme expressing cells.** Estrogen-stimulated cell proliferation in MCF-7/Rz23–9 cells was measured by a colorimetric assay. The OD for cells cultivated in the presence of doxycycline (Rz off) was set as 100%. Mean ± S.E. from three independent experiments done in triplicate is shown. *, $p < 0.05$ *versus* values from doxycycline-treated cells (Student's *t* test).

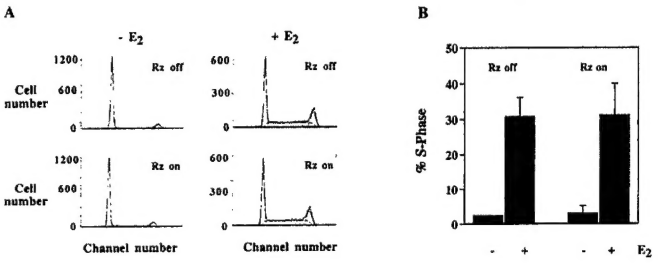

FIG. 5. **Cell cycle progression of MCF-7 cells is not affected by AIB1 levels.** *A*, cell cycle analysis profile of MCF-7/Rz23–9 cells in the presence (Rz off) or absence (Rz on) of doxycycline. Cells were serum-starved for 48 h and then treated for 24 h with 10 nM ICI 182,780 in the absence (*left panels*) or presence (*right panels*) of 100 nM 17β-estradiol. *B*, mean of the percentage of cells in S phase ± S.E. from three independent experiments done in triplicate is shown.

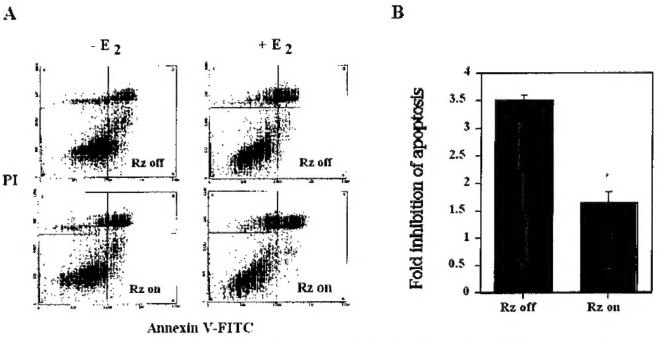

FIG. 6. **Down-regulation of AIB1 levels reduces estrogen-mediated inhibition of apoptosis.** *A*, fluorescence-activated cell sorting analysis of MCF-7/Rz23–9 cells kept in the presence (Rz off) or absence (Rz on) of doxycycline. The cells were stained with fluorescein isothiocyanate (*FITC*)-conjugated annexin V and propidium iodide (*PI*) and analyzed by flow cytometry. Cells were serum-starved for 72 h and then treated for 48 h with 10 nM ICI 182,780 in the absence (*left panels*) or presence (*right panels*) of 100 nM 17β-estradiol (E_2). Cells in the early stages of apoptosis were used for quantitation (shown in *B*) stain for annexin V and are shown in the *lower right quadrant*. *B*, mean of estrogen-mediated inhibition of apoptosis ± S.E. from one representative experiment done in duplicate. *, $p < 0.05$ *versus* values from doxycycline-treated (Rz off) cells (Student's *t* test).

tion of endogenous AIB1 protein levels (see Figs. 1 and 2), we hypothesized that AIB1 could be a rate-limiting factor for estrogen-dependent growth in MCF-7 cells. To test this hypothesis, we first performed cell growth assays. As Fig. 4 demonstrates, when we down-regulated AIB1 in MCF-7/Rz23–9 cells, 17β-estradiol-induced growth was reduced by 50%. A similar effect was observed with the clonal cell line MCF-7/Rz12–9 (data not shown). Since estrogens contribute to cell cycle progression (34, 35) and inhibition of apoptosis in MCF-7 cells (36), we analyzed whether lowered estrogen-mediated growth after AIB1 down-regulation might have resulted from a reduced ability of these cells to progress through the cell cycle or whether this effect might have been based on their altered susceptibility toward apoptosis. When we tested cell cycle progression of MCF-7/Rz23–9 cells after estrogen induction, we detected no significant difference in cell cycle progression dependent on the AIB1 level of these cells (Fig. 5, *A and B*). However, when we challenged MCF-7/Rz23–9 cells by serum starvation, the ability of estrogen to inhibit apoptosis of these cells was strongly reduced in cells in which AIB1 levels were down-regulated (Fig. 6, *A* and *B*). We conclude from these data that AIB1 is essential for estrogen-dependent growth of MCF-7 cells and that reduced growth caused by down-regulation of AIB1 is at least partially due to the reduced ability of estrogen to inhibit apoptosis.

In a separate measure of effects on tumor cell growth, we tested whether anchorage-independent soft agar colony formation of these cells in response to estrogen would also be affected. In the presence of the anti-estrogen ICI 182,780, MCF-7/Rz23–9 cells do not form colonies (Fig. 7, *A* and *C*). However, when these cells are treated with 17β-estradiol, a striking difference between the AIB1-reduced cells (Rz on) and control cells (Rz off) became apparent. Reduction of AIB1 reduced the ability of these cells to form colonies in response to estrogen (Fig. 7, *D versus B* and *E*), indicating a rate-limiting role for AIB1 in estrogen-stimulated anchorage-independent growth of human breast cancer cells.

Subcutaneous Growth of MCF-7 Tumor Cells in Nude Mice—The previous results raised the question whether down-regulation of AIB1 in MCF-7 cells also limits their growth potential in an environment exposed to physiological stimuli from stromal tissue. While MCF-7 cells cultured *in vitro* are exposed to a limited number of autocrine and paracrine growth factors, we wanted to determine whether factors supplied by the host may compensate for lower AIB1 levels in order to stimulate MCF-7 cell growth. We injected MCF-7/Rz23–9 cells subcutaneously into nude mice and followed tumor growth (Fig. 8). Animals in which ribozyme expression was prevented by feeding them a doxycycline-containing diet developed a significantly higher number of tumors relative to controls (6 of 10 *versus* 1 of 8, respectively; $p < 0.05$) (Fig. 8*A*), which was also reflected in a

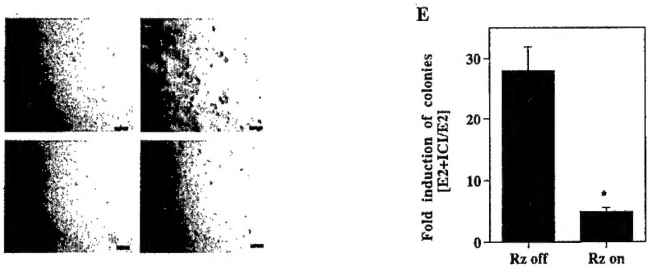

FIG. 7. **Anchorage-independent growth of MCF-7 cells can be inhibited by AIB1 ribozyme targeting.** *A–D*, MCF-7/Rz23–9 cells (*A* and *B*, with doxycycline (Rz off); *C* and *D*, no doxycycline (Rz on)) were cultivated in soft agar in the absence (*A* and *C*) or presence of estrogen (*B* and *D*). Representative images (*A–D*) of colony formation and the mean ± S.E. from one representative experiment done in triplicate (*E*) are shown. *, $p < 0.05$ *versus* values from doxycycline-treated (Rz off) cells (Student's *t* test).

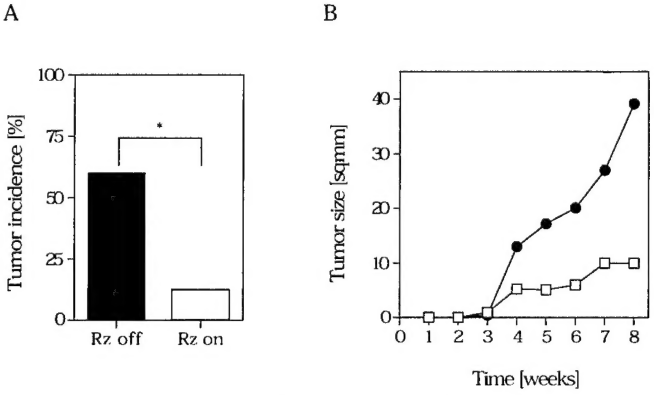

FIG. 8. **Tumor growth of MCF-7/Rz23–9 cells in athymic nude mice.** *A and B*, MCF-7/Rz23–9 cells were injected subcutaneously into the flanks of athymic nude mice at 2×10^7 cells/injection site and 2 sites/animal ($n = 5$ animals in the presence of doxycycline (AIB1 ribozyme blocked); $n = 4$ animals without doxycycline (AIB1 ribozyme active)). *A*, tumor incidence 2 months after injection from animals fed with doxycycline containing diet (Rz off) and from the animals fed with normal diet (Rz on) (*, p value < 0.05 for χ^2 test). *B*, the tumor area was measured every 2–3 days (*closed circles*, Rz off; *open squares*, Rz on), and mean tumor size is shown for 2 months following the injection date.

larger average tumor size relative to controls (Fig. 8*B*). This indicates that neither host factors nor other nuclear receptor cofactors present in MCF-7 cells can compensate for reduction of the nuclear receptor coactivator AIB1 during estrogen-dependent growth *in vivo*.

DISCUSSION

The discovery of specific coactivators and corepressors that modulate the transcriptional activity of the ER and the identification of ER cofactors that are amplified and overexpressed in breast tumors led to the hypothesis that some of these cofactors contribute directly to the development of breast cancer. Some of the best characterized nuclear receptor coactivators to date include CBP/p300 (37, 38) and members of the p160/SRC family including SRC-1 (7), TIF-2 (8), and AIB1 (9) (ACTR/RAC3/TRAM-1/SRC-3) (10–13). Cofactors that are amplified and overexpressed in breast tumors include PBP, ACS2, SRA (39–41), and AIB1. Despite many similarities of these cofactors, which have been shown to bind to the same nuclear receptors and enhance the transcriptional activity of the same receptors *in vitro*, it is hard to predict their function *in vivo*. For example, CBP and p300 both enhance retinoic acid-mediated transcription *in vitro* (37, 42), but they have distinct functions *in vivo* during retinoic acid-induced differentiation of carcinoma F9 cells (43). In addition, functional redundancy of nuclear receptor cofactors might compensate for the complete loss or reduced levels of one of these cofactors as exemplified by a study that showed that SRC-1 potentiates peroxisome proliferator-activated receptor α activity *in vitro* but still was not essential for peroxisome proliferator-activated receptor α-regulated gene expression *in vivo* (44). It is therefore imperative to identify the function of these coactivators directly in a cellular context. So far, despite several reports (9, 16–19, 39–41, 45) demonstrating differential expression patterns of some of these coactivators in breast tumors and defining their interaction with various signaling molecules *in vitro*, the functional role for these cofactors in normal mammary gland development and for breast tumor development is unclear.

In this study, we demonstrate for the first time that ribozyme-targeting of the nuclear cofactor AIB1 reduces estrogen-dependent proliferation and neoplastic growth of human MCF-7 breast cancer cells. Based on these data, we propose that AIB1 overexpression provides a selective advantage for tumor growth in mammary epithelium. This hypothesis is in concordance with the finding that AIB1 amplification in breast tumors correlates with ER and PR positivity and tumor size, as shown in a study based on 1,157 human breast tumors (16). In addition, it has been shown that endogenously expressed human ERα and AIB1 interact in MCF-7 cells (20), supporting the idea that AIB1 could be a rate-limiting factor for estrogen-mediated growth in breast tumor cells. Our results would also suggest that human AIB1 and its mouse homolog p/CIP might have similar functions in the development of mammary epithelium, since deletion of the p/CIP gene showed blunted mammary gland development in the mouse (26). Furthermore, a recent study showed that Taiman, the *Drosophila* homolog of AIB1, contributes to steroid hormone-mediated motility of Drosophila border cells but interestingly has no effect on the proliferation of these cells (46). These findings raise the question of whether there are fundamental differences in the function of these closely related coactivators based on species or tissue context or whether some of these effects might be compensated by functional redundancy of nuclear receptor coactivators.

Previous studies of SRC-1 demonstrate that it is a coactivator of ER (7); however, its role in breast cancer seems less pronounced, and our studies suggest that it is unable to compensate for a loss of AIB1 function. Consistent with this, gene disruption studies of SRC-1 in mice showed only relatively subtle defects in the development of estrogen-dependent tissues, which might be due to the compensatory up-regulation of the related coactivator TIF-2 but not of AIB1 (47). In addition, the highest levels of SRC-1 were found in normal human breast tissue compared with lower levels in breast tumors (45), and in contrast to AIB1, SRC-1 expression did not correlate with ER status of these tumors (45). Furthermore, SRC-1 does not colocalize with the ER in rat mammary epithelium (48); nor did it interact with the endogenous ER in MCF-7 cells (20). Overall, our data and these previous observations indicate a lesser role for SRC-1 in human breast tumorigenesis.

The gene targets of AIB1 in breast cancer are currently not known but may involve its interactions with a number of transcription factors including p53 (23), CBP (10, 12), CARM1 (49), NF-κB (22), and TEF (21) as well as with several members of the nuclear receptor family. It is an interesting possibility that many of these interactions might not only be relevant for breast tumorigenesis but also for a variety of other cancers such as ovarian, pancreatic, and gastric cancer in which AIB1 is also amplified or overexpressed (9, 14, 15). Ribozyme targeting of AIB1 will be a valuable tool to explore this.

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